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Effects of Double-Site Mutations of Vesicular Stomatitis Virus Glycoprotein G on Membrane Fusion Activity

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Site-directed mutagenesis of specific amino acids within a conserved amino-terminal region (H2) and a conserved carboxyl-terminal region (H10/A4) of the fusion protein G of vesicular stomatitis virus have previously identified these two segments as an internal fusion peptide and a region influencing low-pH induced conformational change, respectively. Here, we combined a number of the substitution mutants in the H2 and H10/A4 regions to produce a series of double-site mutants and determined the effect of these mutations on membrane fusion activity at acid pH and on pH-dependent conformational change. The results show that most of the double-site mutants have decreased cell–cell fusion activity and that the effects appeared to be additive in terms of inhibition of fusion, except for one mutant, which appeared to be a revertant. The double-site mutants also had pH optima for fusion that were lower than those observed with wild-type G but same as the pH optima for the parent fusion peptide (H2) mutants. The results suggest that although the H2 and H10/A4 sites may affect membrane fusion independently, a possible interaction between these two sites cannot be ruled out.

INTRODUCTION

Vesicular stomatitis virus (VSV) is a member of the vesiculovirinae subfamily of the Rhabdoviridae family. The single viral glycoprotein G is sufficient to induce cell fusion at acidic pH (Coll, 1995; Florkiewicz and Rose, 1984; Reidel, *et al.,* 1984; Wagner and Rose, 1996; White *et al.,* 1981). Fusion of cell membranes by G protein is induced at ~pH 6.3 with a maximum fusogenic activity observed at pH 5.6. Exposure to acidity is believed to induce a reversible conformational change in the G protein that activates the fusion domain and allows it to be inserted into a target membrane (Blumenthal *et al.,* 1987; Crimmins *et al.,* 1983; Gaudin *et al.,* 1993; Hernandez *et al.,* 1996; Hughson, 1995; Pak *et al.,* 1997; Puri *et al.,* 1988; White, 1990, 1992). Studies from our (Li *et al.,* 1993; Zhang and Ghosh, 1994) and other (Durrer *et al.,* 1995; Fredricksen and Whitt, 1995; Whitt *et al.,* 1990) laboratories have identified within the ectodomain an internal H2 region (amino acids 117–137) as the fusion peptide. Unlike other fusion peptides, the H2 region is not hydrophobic but contains glycine, proline, aspartic acid, and neutral amino acids. It is also highly conserved within the vesiculovirinae subfamily (Bhella *et al.,* 1998), and substitution of conserved glycine, proline, or aspartic acid residues either abolished cell fusion or shifted the pH optimum of fusion to a more acidic value with concomitant reduction in efficiency (Fredricksen and Whitt, 1995; Zhang and Ghosh, 1994). Evidence that the H2 region interacts with the target membrane was provided by hydrophobic photolabeling experiments in which a large peptide segment encompassing residues 65–237 of VSV G was labeled when VSV and liposomes containing the hydrophobic photolabeling crosslinking reagent were exposed to acidic pH (Durrer *et al.,* 1995). Recently, a carboxyl-terminal region, H10/A4, of G protein encompassing residues 395–418, which is also conserved in vesiculovirinae subfamily (Bhella *et al.,* 1998), has been identified as a region influencing conformational changes that induce fusogenic activity (Shokralla *et al.,* 1998). Substitutions of conserved glycine or aspartic residues in this region either abolished or decreased cell–cell fusion, as well as reduced the pH threshold and pH optimum of membrane fusion. The mutants also showed conformational changes as evident by markedly altered resistance to tryptic digestion at acidic pH. The H10/A4 region was, however, not labeled under acidic conditions by hydrophobic crosslinking reagents present in liposomes, indicating that this region is not inserted into the target membrane (Durrer *et al.,* 1995; Gaudin *et al.,* 1995). Recently, amino acids controlling the low-pH-induced conformational change of rabies virus G glycoprotein were shown to be present in a carboxyl-terminal region (Gaudin *et al.,* 1996). Sequence analyses of G protein from two isolated mutants of rabies virus escaping neutralization by monoclonal antibodies recognizing the low-pH-induced form of G showed mutations of Val392 and Met396 to glycine and threonine, respectively (Gaudin *et al.,* 1996). When the rabies virus G protein sequence was aligned with G proteins from a number of rhabodovi-

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ruses, it was observed that the H10 and A4 fusion defective insertion mutants of VSV G protein, as well as the substitution mutants in the H10/A4 segment, were located at the same region (Gaudin *et al.,* 1996; Li *et al.,* 1993; Shokralla *et al.,* 1998). Thus the H10/A4 region may define a domain that controls the low-pH-induced conformational changes leading to fusion.

In this study, we combined a number of the substitution mutants in H2 and H10/A4 regions of VSV G protein to produce a number of double-site mutants and determined the effect of these mutations on membrane fusion activity and on low-pH-dependent conformational change. Double-site mutants have been used previously to study mechanisms of receptor binding (Campion *et al.,* 1993), enzymatic activities (Carter *et al.,* 1984; Mildvan *et al.,* 1992; Wells, 1990), protein denaturation and folding (Shortle, 1995), and acid pH-induced membrane fusion activity of influenza virus haemagglutinin (HA) protein (Steinhauer *et al.,* 1996). Substitution of conserved residues in the H2 fusion domain or in the H10/A4 region, affecting the low-pH-induced conformational change, indicate that the mutations affect fusion activity. It is possible that the individual mutations disrupts specific interactions involving the parent regions and that any structural alterations arising from the mutation affect only the site of mutation. However, the mutation at one site can also affect a second site either directly or indirectly through structural changes induced. To assess whether mutations in the two sites function independently or mutation at one site alters the effect of the second site in the molecule, a series of double-site mutants involving H2 and H10/A4 regions were constructed. The results indicated that the decrease in fusogenic activities of the double-site mutants appear mostly to be additive, except for a double mutant, which behaved as a revertant. However, the optimum pH of fusion of the double mutants corresponded in most cases to those observed for the parent fusion peptide mutants. These results suggest that although the two sites H2 and H10/A4 may function independently, interactions between these sites may be possible.

TABLE 1

Summary of the Properties of H2, H10/A4, and the Double-Site Mutants

G protein	Cell surface expression iodination[a] (%)	pH optimum of fusion[c]	pH threshold of fusion[d]	Fusion activity at optimum pH[b] (%)
Wild-type	100	5.6	6.3	100
H2 mutants				
F125Y	91	5.2	6.0	34
P126L	66	5.4	6.0	48
G131A	120	5.0	6.0	70
D137N	126	5.0	6.0	48
H10/A4 mutants				
G395E	98	5.0	5.8	18
G404A	158	5.6	6.0	42
D411N	121	5.5	6.0	46
Double mutants				
F125YD411N	21	—[e]	—	1
P126LD411N	57	—	—	5
D137ND411N	199	—	—	<1
G131AD411N	150	5.0	6.0	24
G131AG395E	148	5.0	5.8	28
G131AG404A	123	5.0	6.3	83
D137NG404A	118	5.0	5.4	18

[a] For quantification of cell surface expression, COS cells transfected with wild-type or mutant G plasmids were iodinated with ^{125}I in a lactoperoxidase-catalyzed iodination reaction at 24 h posttransfection. The results shown are the averages of two separate experiments.

[b] Cell–cell fusion at optimum was determined as from Fig. 4.

[c,d] The pH optima and thresholds of cell–cell fusion were determined by counting polykaryons over a pH range of 4.8–6.3.

[e] Indicates that the corresponding mutant glycoprotein is fusion defective over the pH range of 4.8–6.3.

RESULTS

Construction of double-site mutants and expression of the mutant G proteins

Substitution of conserved residues in the H2 and H10/A4 regions of VSV G protein resulted in the abolition or decrease in fusion activity with concurrent shifts to more acidic pH values for the pH optima and threshold of fusion (Fredericksen and Whitt, 1995; Shokralla *et al.,* 1998, Zhang and Ghosh, 1994). Mutants with reduced fusogenic activities were selected to construct double-site mutants. A summary of the fusogenic efficiency, as well as the pH optima and threshold of fusion of the mutants, are shown in Table 1. We reported earlier that within the fusion peptide, substitution of Gly124 or Pro127 led to almost complete inhibition of fusion. These were therefore not used to construct double-site mutants. Residues Phe125, Pro126, Gly131, and Asp137 were all conserved in the vesiculovirinae subfamily. Substitution mutants, such as F125Y, P126L, G131A, and D127N, showed maximum fusion activities of 34%, 48%, 70%, and 48%, respectively, at their corresponding optimum pH of fusion (Zhang and Ghosh, 1994, unpublished). The pH optima and the threshold pH for fusion were shifted to more acidic values compared with wild-type G protein. Substitution of conserved residues Gly406 or Asp409 in the H10/A4 region caused almost complete inhibition of fusion. The mutants G395E, G404A, and D411N, which showed maximum fusion efficiency of 18%, 42%, and 46%, were chosen for the second-site mutation. As in the case of fusion peptide mutants, all of these mutants also showed a decrease in both pH optima and threshold of fusion (Shokralla *et al.,* 1998). A series of double-site mutants of the fusion peptide and the region influencing conformational change were constructed and are shown in Table 1. The presence of conserved glycine and acidic amino acids in the

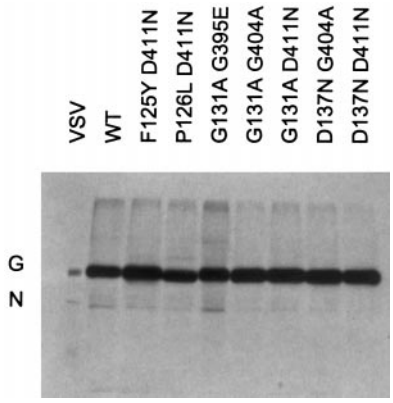

FIG. 1. Expression of double-site mutant G genes. COS cells were transfected and labeled with ^{35}S-methionine, and the cell lysates were immunoprecipitated with anti-G antibody and analyzed by SDS–PAGE. Mutants are indicated above the lanes. WT, wild-type, VSV marker-G, 67,000 Da; N, 50,000 Da.

fusion peptide has been shown to be important for the fusogenic activities of viral glycoproteins requiring acid pH for membrane fusion (Fredricksen and Whitt, 1995; Gething *et al.,* 1985; Hernandez *et al.,* 1996; Levy-Mintz and Kielian, 1991; Ohnishi, 1988; Skehel *et al.,* 1995; Steinhauer *et al.,* 1995; Zhang and Ghosh, 1994). Not only was Asp411 conserved in a number of vesiculoviruses, but most of the rhabdoviruses contained an acidic amino acid at this position in G protein (Gaudin *et al.,* 1996; Shokralla *et al.,* 1998).

The double-site mutant genes were expressed in COS cells using a high-expression pXM vector (Li *et al.,* 1993; Yang *et al.,* 1986), and proteins labeled with ^{35}S-methionine were analyzed by immunoprecipitation with a polyclonal anti-G antibody followed by SDS–PAGE. All seven mutant G constructs expressed proteins that comigrated with wild-type G protein (Fig. 1). The intensities of bands corresponding to the wild-type and the double-site mutants were similar, suggesting that the substitution of conserved residues in two separate sites involved in G protein-mediated membrane fusion did not affect the expression of the protein.

Intracellular localization and transport of double mutants

Cell–cell fusion requires the localization of the fusion protein at the cell surface. To determine whether simultaneous mutations at the H2 and H10/A4 sites affected the intracellular transport and cell surface localization, COS cells expressing wild-type and mutant G proteins were examined by indirect immunofluorescence. Wild-type G and the five mutants G131AG395E, G131AG404A, G131AD411N, D137NG404A, and D137ND411N all showed strong cell surface fluorescence, with intensities dependent on the amount of DNA used in transfection (Fig. 2). The mutants F125YD411N and P126LD411N, however, showed much more diminished cell surface fluorescence. Examination of the internal immunofluorescence of these two mutants showed strong internal labeling, which was comparable to the internal fluorescence of wild-type G protein as well as that of the other mutants (data not shown), suggesting that the decreased cell surface expression of F125YD411N and P126LD411N mutants was not due to reduced expression of the proteins.

The distribution of the mutant G proteins at the cell surface was determined quantitatively using lactoperoxidase-catalyzed cell surface iodination (Guan *et al.,* 1985; Li *et al.,* 1993). The relative amounts of the proteins labeled on the cell surface were quantified by densitometric scanning of the fluorograms. The amounts of mutant G proteins present on the cell surface were 100–200% of the wild-type G protein depending on the amount of DNA used for transfection (Table 1). However, the two mutants F125YD411N and P126YD411N showed a decreased amount of ~20% and ~60%, respectively, of the wild-type G protein present in the cell surface. This is in agreement with the weak cell surface immunofluorescence observed in the case of these two mutants (Fig. 2); this suggests that the intracellular transport of these two mutants may be affected by double-site substitutions.

Transport of the proteins within the cell, from the endoplasmic reticulum to the Golgi complex, was evaluated by the acquisition of endo H resistance (Kornfeld and Kornfeld, 1985). The results presented in Fig. 3A show that after a 15-min pulse, all seven double-site mutants were sensitive to endo H, showing that they were glycosylated with *N*-linked oligosaccharides. After a 1-h chase (Fig. 3B), mutants G131AG395E, G131AG404A, G131AD411N, D137ND411N, and D137NG404A were 100% resistant to endoglycosidase H (endo H) digestion, indicating transport from the endoplasmic reticulum to the Golgi complex. Mutants F125YD411N and P126LD411N were, however, 65% and 80%, respectively, resistant to digestion at 1 h, suggesting impaired intracellular transport, possibly due to structural alterations. It should also be noticed that in the cases of these two mutants, the amounts of the mutant proteins after a 1-h chase is not reduced.

Fusion characteristics of double-site mutant G proteins

Cellular membrane fusion involves mixing of the individual cell contents as a result of fusion of cell membranes to form multinucleated cells. Cells expressing VSV G protein at the cell surface can fuse to form polykaryons when exposed to acidic pH for a very short time (Florkiewicz and Rose, 1984; Li *et al.,* 1993; White *et al.,* 1981). Cells expressing wild-type G protein showed extensive cell fusion at pH 5.6, and the polykaryons formed contained 20–50 nuclei. The mutants

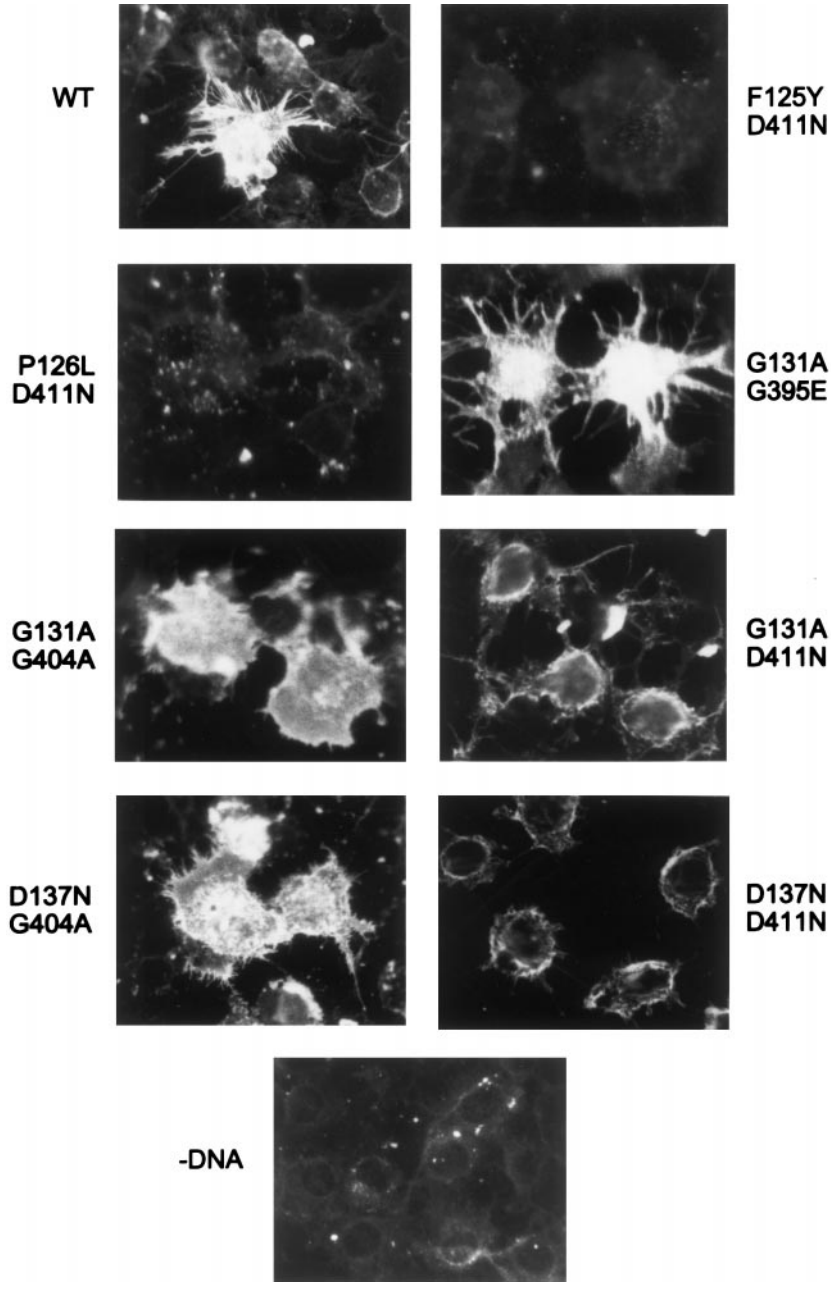

FIG. 2. Cell surface localization of wild-type and mutant G proteins by immunofluorescence. Transfected COS cells were fixed with paraformaldehyde. The proteins were detected using rabbit anti-G antiserum and fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G.

G131AG404A, G131AD411N, G131AG395E, and D137NG404A showed polykaryon formation at pH 5.6. The number of polykaryons except for G131AG404A were 1–10% of that observed for wild-type G. As well, the number of nuclei present in these polykaryons were fewer. The mutants P126LD411N, D137ND411N, and F125YD411N hardly showed any polykaryons at pH 5.6 (data not shown).

Studies with mutants in the fusion peptides of influenza virus HA protein or Semliki Forest virus E1 protein have shown either an increase or a decrease in the pH optimum required for membrane fusion (Levy-Mintz and Kielian, 1991; Skehel *et al.,* 1995; Steinhauer *et al.,* 1995). We (Zhang and Ghosh, 1994) and others (Fredericksen and Whitt, 1995) have also reported that substitution of amino acids in the fusion peptide domain of VSV G protein also shifted the pH optima and threshold of fusion to more acidic values. Similar shifts of pH optima were also observed in the case of chimeric G proteins containing foreign transmembrane and cytoplasmic domains (Odell *et al.,* 1997), as well as mutants in the H10/A4 domain (Shokralla *et al.,* 1998). Studies with double mutants of influenza virus HA protein further showed that the effect of substitution of amino acids at two sites could be additive or nonadditive (Steinhauer *et al.,*

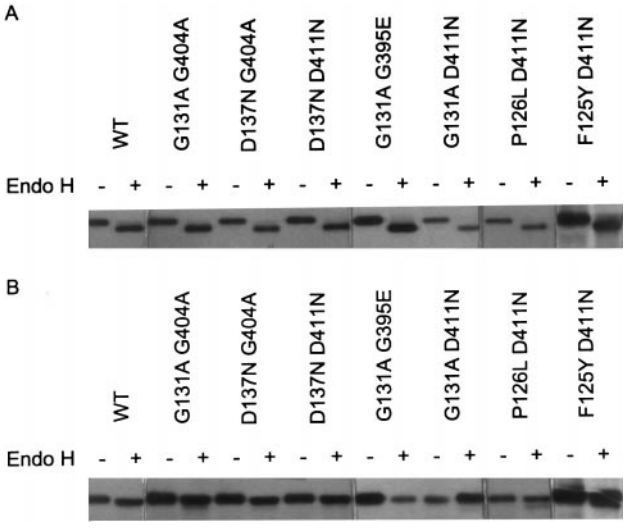

FIG. 3. Acquisition of endo H resistance by wild-type and mutant G proteins. COS cells transfected with wild-type or mutant constructs of the G protein were labeled at 24 h posttransfection with ^{35}S-methionine for 15 min and chased with an excess of nonradioactive methionine for a period of 0 min (A) or 60 min (B). After immunoprecipitation with anti-G antibody, one half of the sample was treated with endo H (+). and the other half was not (−). Samples were analyzed by SDS–PAGE. Extent of endo H resistance acquired was determined by densitometric scanning of the fluorograms.

1996). The pH dependence of the double-site mutants of VSV G protein were therefore examined over the pH range of 4.8–6.3. Results presented in Fig. 4 show that initial fusion occurs at pH 6.3 for both wild-type G and mutant G131AG404A. The optimum pH for fusion for mutant G131AG404A was shifted to a more acidic pH of 5.0, corresponding to the parent H2 mutant. The efficiency of fusion at the optimum pH was only slightly reduced compared with wild-type G. Thus this mutant behaved similar to wild-type except for its requirement of more acidic pH for optimum fusion. The mutant G131AG404A showed a relative fusion efficiency of 83% compared with 70% and 40% efficiency observed with the parent mutants G131A and G404A, respectively, suggesting that mutation in the one site is compensating the inhibitory effect of the mutation in the other site, presumably by perturbing the structure in such a way that wild-type activity is partially restored. The three mutants G131AD411N, G131AG395E, and D137NG404A are also compromised in their ability to induce fusion, with a ~70–80% reduction in syncytia formation compared with wild-type G. These mutants showed maximum fusion at pH 5.0, corresponding to the optimum pH of fusion of the parent H2 mutant. The pH threshold of fusion is, however, shifted toward a more acidic pH compared with wild-type G. The values of threshold pH of fusion for mutants G131AD411N and G131AG395E remain the same as those corresponding to the parent H10/A4 mutants, whereas mutant D137NG404A requires a more acidic pH than either parent H2 or H10/A4 mutants. The fusion activity of these three double mutants appears to be additive; for example, mutant G protein containing G131A and D411N amino acid substitutions that individually reduced the fusion activity by 30% and 54%, respectively, showed a fusion efficiency of 24%. The two double mutants P126LD411N and D137ND411N show severely reduced fusion over the entire range of pH tested. The observed loss of fusion activity of the mutant F125YD411N can also be related to its impaired surface expression (Table 1).

Oligomerization of the mutant G proteins

Low-pH-induced membrane fusion by influenza virus HA glycoprotein and Semiliki Forest virus envelope gly-

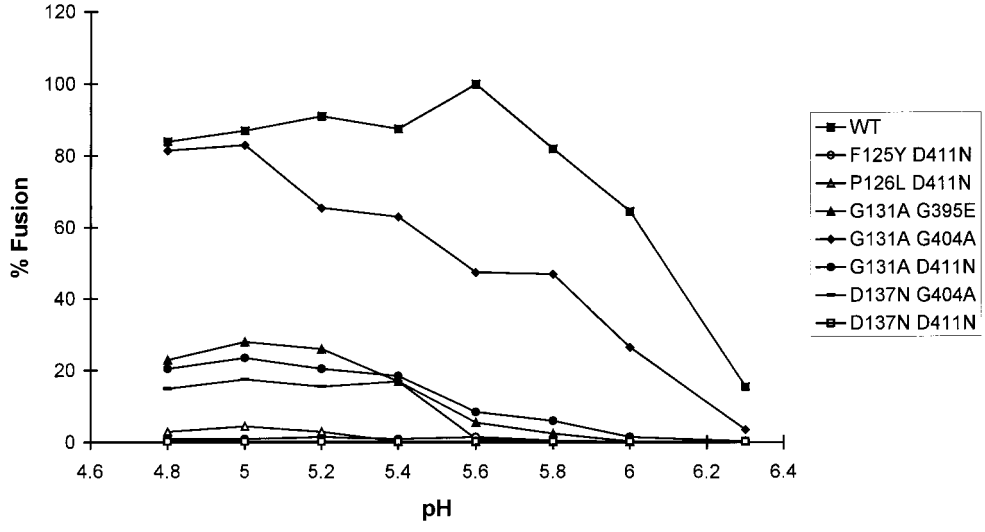

FIG. 4. The pH dependence of cell fusion induced by expression of mutant G proteins. COS cells transfected with wild-type or mutant G plasmids were exposed to fusion media of varying acidity of pH 4.8–6.3 as described for the polykaryon formation assay. The total number of polykaryons produced by wild-type G protein at pH 5.6 was taken as the standard measure of 100%. The data shown are the average of two separate experiments.

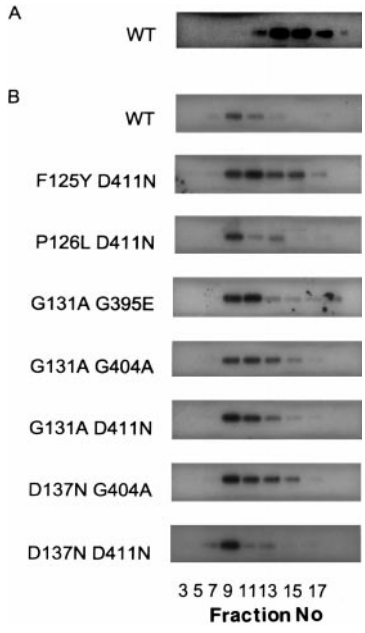

FIG. 5. Oligomer formation of mutant and wild-type G proteins. COS cells transfected with wild-type or mutant constructs were labeled at 24 h posttransfection with ^{35}S-methionine and chased with excess nonradioactive methionine. Cells were lysed in 4× MNT containing 1% Triton X-100, and the lysates were loaded onto a 5–20% sucrose gradient. Fractions were collected from the bottom, immunoprecipitated with anti-G antibody, and analyzed by SDS–PAGE. The bottom fraction is to the left. 8S (aldolase) and 4S (BSA) markers sedimented in fractions 9 and 13, respectively. (A) Wild-type G sample analyzed at pH 7.4. (B) Samples analyzed at pH 5.4.

coprotein E1 required homotrimerization of the fusion proteins (Hernandez *et al.,* 1996; Kielian, 1995; Skehel *et al.,* 1995; Wahlberg *et al.,* 1992). VSV G protein also forms a trimer that is essential for its transport to the cell surface (Kreis and Lodish, 1986). The G protein trimer, however, is more stable at acidic pH than at neutral pH (Doms *et al.,* 1993, 1988). To evaluate whether the double mutations affected the oligomerization of mutant G proteins, and hence their transport and fusogenic activity, we determined the oligomeric structure of the double mutants by sucrose density gradient centrifugation at acidic pH (Crise *et al.,* 1989). As reported, the wild-type G protein formed trimers at pH 5.5, whereas at pH 7.4, it sediments as a monomer (Fig. 5). Double-site mutant G proteins showed sedimentation patterns that are similar to those of wild-type G protein at acidic pH (Fig. 5). However, it should be noted that in the case of mutants F125YD411N and D137NG404A, dimers and monomers were also present in the gradient, suggesting that the trimeric forms of these two mutants may not be as stable as the wild-type G protein and thus tend to dissociate even at pH 5.5. Thus the structural perturbations created by the second-site mutations may affect the folding and oligomerization that are reflected in the altered transport and fusion defects for these two mutants. Other mutants formed trimers as well as the wild-type G protein, suggesting that the altered fusogenic activities of these mutants were not due to incorrect oligomerization.

Conformational changes in double-site G mutants at acid pH

The fusion glycoproteins of rhabdoviruses, such as VSV or rabies virus, require activation from a nonfusogenic to a fusogenic state that is induced by a reversible pH-dependent conformational change (Blumenthal *et al.,* 1987, Gaudin *et al.,* 1995, 1993, Pak *et al.,* 1997). It was previously shown that VSV G protein becomes resistant to tryptic digestion at low-pH, presumably due to conformational change or changes induced by acidic pH (Fredericksen and Whitt, 1996; Odell *et al.,* 1997; Shokralla *et al.,* 1998). To investigate whether the changes in the fusogenic characteristics of the double mutants of G protein were due to alterations in the low-pH-induced conformational change, we determined the pH-dependent resistance to typtic digestion of mutant and wild-type G proteins. Results presented in Fig. 6 show that the wild-type and the mutant G proteins except G131AG404A and G131AD411N were completely digested by trypsin at neutral pH. The two mutants were ~20% resistant to tryptic digestion at pH 7.4. The wild-type G protein became ~90% resistant to tryptic digestion at pH 6.5, whereas the mutants showed different extents of resistance to tryptic digestion at acidic pH. The mutant G131AG395E was also ~90% resistant at pH 6.5. In contrast, the fusion-incompetent and transport-impaired mutant F125YD411N was completely susceptible to digestion at pH 6.5. Mutants G131AD411N, P126LD411N, and D137ND411N were 50–60% resistant, whereas mutants G131AG404A and D137NG404A were ~30–35% resistant to trypsin digestion at pH 6.5. All of the double mutants became increasingly resistant to trypsin with decreasing pH, and at pH 5.6, they were nearly as resistant as wild-type G. A comparison of the tryptic resistance pattern of the parent H10/A4 mutants with that of the corresponding double-site mutant also showed that in the case of mutants P126LD411N, G131AG395E, and G131AD411N, the extent of resistance at pH 6.5 was similar to the values observed with the parent H10/A4 mutants. In the case of mutants G131AG404A and G137NG404A, the resistance to tryptic digestion at pH 6.5 was reduced, whereas in the case of D137ND411N, which was totally fusion incompetent, the extent of resistance to trypsin digestion was increased compared with the parent H10/A4 mutants. The transport-impaired mutant F125YD411N showed a drastic change in the resistance pattern compared with its parent H10/A4 mutant.

DISCUSSION

Negative-stranded-RNA containing enveloped viruses can fuse cell membranes at both acid and neutral pH (Hernandez *et al.,* 1996; White, 1990, 1992). The mechanism of membrane fusion at acid pH has been most

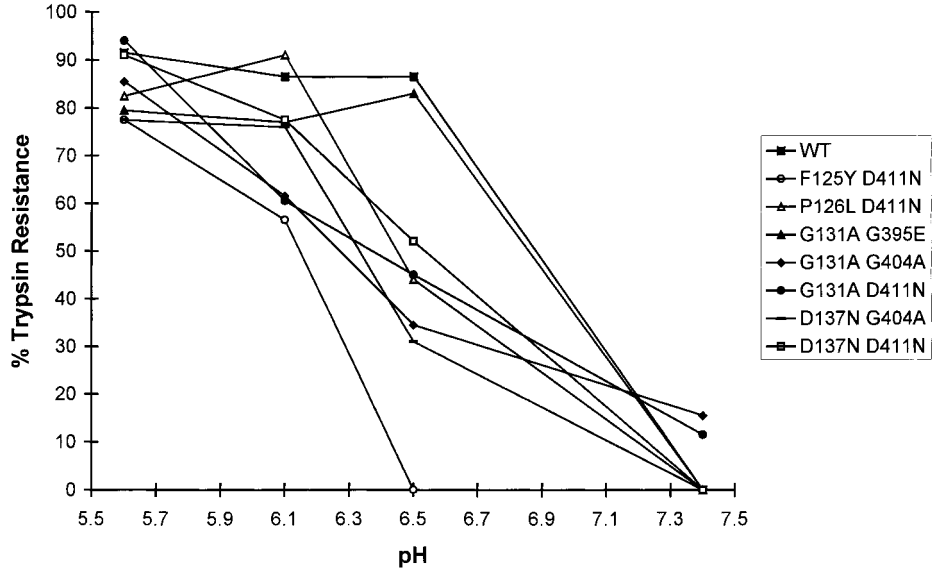

FIG. 6. The pH-dependent resistance of wild-type and mutant G proteins to tryptic digestion. COS cells transfected with wild-type or mutant plasmids were labeled at 24 h posttransfection with ^{35}S-methionine for a period of 30 min and chased with excess nonradioactive methionine for a period of 1 h. The cells were lysed in 2× MNT containing 1% Triton X-100 at the indicated pH, and equivalent amounts of each sample were subjected to tryptic digest. Proteins were immunoprecipitated with anti-G antibodies and analyzed by SDS–PAGE. The relative amounts of G protein that are resistant to digestion at a specific pH were determined using densitometric scanning of a fluorogram, and the percentage trypsin resistance was calculated. The data shown are the average of two separate experiments.

extensively studied with influenza virus (Carr *et al.,* 1997; Gaudin *et al.,* 1995; Hernandez *et al.,* 1996; Hughson *et al.,* 1995; Skehel *et al.,* 1995), particularly because of the availability of the three-dimensional structure of its HA fusion protein (Bullough *et al.,* 1994; Skehel *et al.,* 1995). Although the three-dimensional structure of VSV G protein is not known, a large number of biochemical, biophysical, and molecular genetic studies show that the characteristics of G protein and its fusion process is different: (1) Unlike most viral fusion proteins VSV G is not proteolytically cleaved. (2) The fusion peptide of VSV G is not localized at the N-terminus but instead is internal. (3) The fusion peptide is not hydrophobic in nature but instead contains neutral amino acids. (4) The acid-induced conformational change of G protein required for membrane fusion is reversible such that the fusogenic form reverses back to the native nonfusogenic form after reneutralization, suggesting that G protein may not be in a metastable form at the native state (Blumenthal *et al.,* 1987; Carr *et al.,* 1997; Durrer *et al.,* 1995; Gaudin *et al.,* 1995; Pak *et al.,* 1997; Puri *et al.,* 1988; Wagner and Rose, 1996; Zhang and Ghosh, 1994). The present study was directed to examine whether the membrane insertion peptide (H2 region) and the region controlling the acid-induced conformational change (H10/A4 region) of VSV G protein act independently or interact with each other or if one region dominates over the other. Double-site mutants in which conserved amino acids in both the H2 and H10/A4 regions were substituted simultaneously and the effects on fusion and conformational change were studied.

It is generally believed that single substitution by conservative or semiconservative amino acids can cause local perturbations in structure that may affect protein activity either directly or indirectly when a mutated residue makes contact with another residue. Simultaneous substitution of two residues at different regions of a protein may independently cause local perturbations in structure such that the observed effect on activity is additive (Wells, 1990). However, in some cases, the activity of the double mutant is not simply additive, indicating that the mutated residues may interact with each other either directly or indirectly via electrostatic interactions or structural perturbations. In these cases, the mutations in the two sites do not act independently (Campion *et al.,* 1993; Wells, 1990). The double mutant D137ND411N did not show any fusion activity over the entire acid pH range tested even when expressed at 200% of the wild-type level. The observed loss in fusogenic activity for the double-site mutant D137ND411N appears to be simply additive of the decrease in fusion activity observed with the individual single-site mutants, suggesting that the two sites H2 and H10/A4 may be acting independently. The double mutants F125YD411N and P126LD411N were impaired in their cell surface localization and in intracellular transport. Both of them did not show membrane fusion activity over the acid pH range of 6.3–4.8 as tested. The observed loss in fusogenic activity for the mutants F125YD411N and P126LD411N could simply be due to their poor expression at the cell surface. The reduced endo H sensitivity suggests that these two mutants may have altered fold-

ing that reduced their transport to the cell surface. The fact that the mutant F125YD411N is totally sensitive to tryptic digestion at pH 6.5 further shows that the structure of this mutant must be altered such that it requires lower pH to induce the conformational change. This is further corroborated by the observed oligomerization pattern at pH 5.6, which suggests that at pH 5.6, trimers formed by F125YD411N may be less stable, leading to inefficient cell surface localization.

The two mutants G131AD411N and D137NG404A also show inhibition of fusogenic activity that appears to be simply additive of the inhibition of fusion observed with H2 and H10/A4 single-site mutants, suggesting that the two regions may be independent in their function. In the cases of the mutants G131AG395E and G131AG404A, the inhibition of fusion observed is less than the simple additive values of inhibition of fusion observed with the parent H2 and H10/A4 mutants, which suggests that in these cases, the two sites may interact or affect each other. The loss of fusion activity caused by one mutant could be compensated by mutation at the second site. The second mutation may have restored the fusion activity by reinstating the interactions essential for efficient fusion. Thus although the mutants G131A and G404A individually reduce the fusogenic activity by 30% and 60%, respectively, the double mutant G131AG404A appears to be a revertant with 83% fusion activity. It should, however, be noted that in the case of the mutant G131AG395E, the observed gain in fusion activity is much less than that observed in the case of the revertant mutant G131AG404A.

The additive or nonadditive effects on the fusogenic activities of the double mutants were also examined by comparing the changes in pH threshold and pH optimum of fusion with respect to their parent single mutants. The pH thresholds of mutants G131AD411N and G131AG395E were the same as the threshold pH values observed with the corresponding H10/A4 mutants. The pH threshold of mutant D137NG404A, however, exceeded the sum of the differences in the pH threshold of the parent H2 and H10/A4 mutants from the wild-type, suggesting the requirement for increased acidity to overcome barriers preventing fusion. The mutant G131AG404A, which was ~80% as active in fusion as the wild-type G protein, showed a reversion of the threshold pH of fusion to the wild-type value of pH 6.3. However, the pH optimum for fusion of this mutant was identical to that of this parent fusion peptide mutant G131A. Indeed, an examination of the fusion profile of the double mutants revealed that each of the fusion competent double mutants tested showed optimum pH 5.0, which corresponded to the pH optima of the parent fusion peptide mutant. It appears, therefore, that the fusion peptide is a dominant determinant for the pH optimum of fusion of VSV G protein. Studies with double mutants of influenza virus HA proteins also indicated that the effects of mutations near the fusion peptide region override those of mutations in other regions (Steinhauer *et al.,* 1996).

The double-site mutants used in this study involved substitution of conserved glycine and/or aspartic acids in either H2 or H10/A4 regions. Conversed glycine residues have been identified as important residues in the fusion peptides present in the fusion proteins of influenza virus (Gething *et al.,* 1986; Steinhauer *et al.,* 1995), human immunodeficiency virus and simian immunodeficiency virus (Bosch *et al.,* 1989; Freed and Martin, 1995), paramyxoviruses (Lamb, 1993; Sergel-Germans *et al.,* 1994), alpha viruses (Kielian, 1995, Levy-Mintz and Kielian, 1992), and VSV (Fredricksen and Whitt, 1995; Hernandez *et al.,* 1996; White, 1990, 1992; Zhang and Ghosh, 1994). Fusion proteins that require acidic pH for membrane fusion, such as influenza virus HA, Semliki Forest virus E1, and VSV G protein, also contained conserved aspartic acids in their fusion peptide region (Fredricksen and Whitt, 1995; Gething *et al.,* 1986; Hernandez *et al.,* 1996; Kielian, 1995; Levy-Mintz and Kielian, 1992; Steinhauer *et al.,* 1995; White, 1990, 1992; Zhang and Ghosh, 1994). The importance of conserved glycine and aspartic acid residues in the H10/A4 domain that controls the acid pH-induced conformational change of VSV G protein has recently been established (Shokralla *et al.,* 1998). Our studies with double-site mutants show that the substitution of conserved glycine and aspartic acid residues in either the H2 or H10/A4 sites modifies the fusogenic activity in an additive or nonadditive fashion. Three fusion-defective double mutants had aspartic acid 411 in the H10/A4 region substituted by a noncharged asparagine. The presence of an acidic amino acid at this position of G protein from all rhabdoviruses with exception of spring viremia of carp virus (Coll, 1995; Gaudin *et al.,* 1996; Shokralla *et al.,* 1998) suggests that this acidic residue must be a key determinant of the fusogenic activity. The perturbation induced by substitution of this acidic residue in the H10/A4 region with a noncharged residue acts additively with the altered structure resulting from mutation of different residues present in the fusion peptide, such as Phe 125, Pro126, Gly131, and Asp137.

Taken together, the results obtained with substitution mutants in the fusion peptide region and in the carboxyl-terminal H10/A4 region, which influences acid pH-induced conformational change required for fusion, suggest that these two widely separated regions are independent in their function. The reversion of both the fusogenic activity and pH of threshold of fusion for the double mutant G131AG404A, however, suggest that these two regions may also interact either directly or indirectly to affect the fusion activity. This may occur through the proposed interaction between the two predicted heptad repeats of rhabdoviral G proteins (Coll, 1995a; Shokralla *et al.,* 1998). The predicted amino terminal heptad repeat follows the fusion peptide, whereas

the carboxyl-terminal heptad repeat is upstream of the H10/A4 region. These two regions could interact presumably via a trimeric coiled-coil structure in an antiparallel fashion that results in rearrangement of the G protein that allows the viral and target cell membranes to be in close proximity and the exposed fusion peptide to be inserted into the cell membrane. It has recently been shown that a number of viral fusion proteins, including those of influenza (Bullogh *et al.,* 1994; Carr and Kim, 1993; Carr *et al.,* 1997; Quiao *et al.,* 1998), retrovirus (Blacklow *et al.,* 1995; Chan and Kim, 1998; Chan *et al.,* 1997; Fass *et al.,* 1996; Lu *et al.,* 1995; Weissenhorn *et al.,*1997), filovirus (Weissenhorn *et al.,* 1998), and paramyxovirus (Joshi *et al.,* 1998; Sergel-Germans *et al.,* 1994; Yao *et al.,* 1996; Young et al., 1997) contain or are predicted to contain coiled-coil regions that are involved in the initiation of fusion. In the case of influenza, retrovirus, and filovirus, crystal structures of proteolytic fragments containing the heptad repeat regions have indeed confirmed this proposed architectural feature.

MATERIALS AND METHODS

Construction of mutant G protein

The gene encoding G protein of VSV Indiana serotype was cloned into the eukaryotic expression vector pXM to produce the plasmid pXM G as described earlier (Li *et al.,* 1993; Shokralla *et al.,* 1998). VSV G protein mutants F125Y, P126L, G131A, and D137N were constructed as described earlier (Zhang and Ghosh, 1994). Mutants G395E, G404A, and D411N were constructed as described previously (Shokralla *et al.,* 1998). The double-site mutants were constructed using *Bgl*II and *Kpn*I restriction endonucleases by subcloning a 1115-bp fragment of pXMG (F125Y, P126L, G131A, or D137N) containing the H2 mutations into the 5681-bp fragment of pXMG (G395E, G404A, or D411N) carrying the H10/A4 mutations. Inserts were sequenced to verify the presence of desired H2 mutations.

Transfection, labeling, and immunoprecipitation

Subconfluent monolayers of COS-1 cells were transfected by the $Ca_3(PO_4)_2$ method, as described earlier (Shokralla *et al.,* 1998). The transfected cells were labeled with ^{35}S-methionine at 24 h posttransfection and processed for immunoprecipitation and analysis by SDS–PAGE as described previously (Shokralla *et al.,* 1998).

Cell–cell fusion assay

The fusogenic activity of the expressed wild-type and mutant G proteins was determined by exposing cells to the indicated pH for a very short time and observing polykaryon formation as described previously (Li *et al.,* 1993; Shokralla *et al.,* 1998). Polykaryons containing more than five nuclei were counted.

Localization and intracellular transport

The cell surface localization of the expressed glycoproteins was examined by indirect immunofluorescence of transfected cells fixed with paraformaldehyde and reacted successively with rabbit anti-G antibody and goat anti-rabbit IgG conjugated to fluorescein as described previously (Shokralla *et al.,* 1998). For quantification of G protein expressed on the cell surface, labeling using lactoperoxidase-catalyzed iodination was carried out as described previously (Guan *et al.,* 1985; Li *et al.,* 1993). Transport of the mutant G proteins to the Golgi apparatus was determined by acquisition of endo H resistance as previously described (Kornfeld and Kornfeld, 1985; Shokralla *et al.,* 1998).

Oligomerization and trypsin sensitivity assays

The oligomeric state of the expressed G protein was determined by sucrose gradient centrifugation as described previously (Crise *et al.,* 1989; Shokralla *et al.,* 1998). Trypsin sensitivity of the wild-type and mutant proteins were determined as described previously (Fredricksen and Whitt, 1996; Odell *et al.,* 1997). Briefly, transfected cells were labeled with ^{35}S-methionine and lyzed with 1% Triton X-100 in 2× MNT [40 mM 2-(*N*-morpholino)ethanesulfonic acid, 60 mM Tris, 200 mM NaCl, 2.5 mM EDTA] buffer at the indicated pH. The lysate was centrifuged at 14,000*g* for 5 min, and equivalent volumes of the supernatant were incubated in the absence or the presence of 10 μg of TPCK-trypsin for 30 min at 37°C. The digestion was stopped by the addition of aprotinin (10 units), and the mixture was centrifuged again at 14,000 rpm for 2–5 min to remove any insoluble material. The supernatant was immunoprecipitated with anti-G (Indiana) antibody and analyzed by SDS–PAGE as described previously (Odell *et al.,* 1997).

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